PA 1237539

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

October 26, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *60/510,231*
FILING DATE: *October 09, 2003*

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS
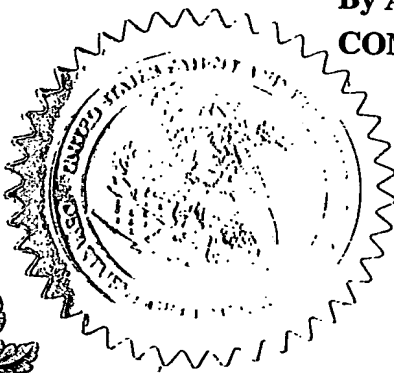
TRUDIE WALLACE
Certifying Officer

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Lab I N .   EL824646997US

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Johnsson	Kai	Lausanne, Switzerland
Tafelmeyer	Petra	Lausanne, Switzerland

☐ Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

TWO HYBRID SYSTEM FOR THE IDENTIFICATION OF FRAGMENTATION SITES IN A PROTEIN

CORRESPONDENCE ADDRESS

Direct all correspondence to:

☑ Customer Number   35,246   ⟶   Place Customer Number Bar Code Label here

Type Customer Number here

OR

☐ Firm or Individual Name	
Address	
Address	
City	State	ZIP
Country	Telephone	Fax

ENCLOSED APPLICATION PARTS (check all that apply)

☑ Specification *Number of Pages* 16

☑ Drawing(s) *Number of Sheets* 4

☐ Application Data Sheet. See 37 CFR 1.76

☐ CD(s), Number

☑ Other (specify) - Return Postcard

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.

☑ A check or money order is enclosed to cover the filing fees

☐ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number:

☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($)

$80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE _____   Date 10/09/2003

TYPED or PRINTED NAME Sherman D. Pernia

TELEPHONE 281-996-0449

REGISTRATION NO. *(if appropriate)*   34,404

Docket Number: JM-213

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

CERTIFICATE OF EXPRESS MAIL DEPOSIT
Under 37 CFR § 1.10

U.S.P.S. EXPRESS MAIL LABEL No: __EL 824646997 US__

DEPOSIT DATE: __9 October 2003__

I, __Sherman D. Pernia__, hereby certify that this correspondence is being deposited with the United States Postal Service using "Express Mail, Post Office to Addressee" service on the date indicated above and is addressed to "COMMISSIONER FOR PATENTS, P.O. Box 1450, Alexandria, VA 22313-1450."

Signed: _____   Docket No. __JM-213__

5

10

United States Provisional Patent Application

TWO HYBRID SYSTEM FOR THE IDENTIFICATION
OF FRAGMENTATION SITES IN A PROTEIN

Inventors:

Kai Johnsson

Petra Tafelmeyer

15

Fig. 1 Identification of fragmentation sites in TRP1 by random circular permutation and homologous recombination.

Fig. 2 Schematic sequences of 13 yPRAI clones isolated after circular permutation and selection for complementation of tryptophan auxotrophy. The green rectangle illustrate the antiparallel leucine zippers, the red rectangles the yPRAI fragments. Two of the 15 clones sequenced that are not shown in the figure had an inverted sequence. However, after homologous recombination active full-length protein could be expressed from the galactose promoter in the inverse direction.

Fig. 3 Sequence alignment of ePRAI, yPRAI and tPRAI. The last residues of each sequence line are numbered. The position and numbering of secondary elements (α-helices and β-strands) for ePRAI and tPRAI are shown as arrows above and below the sequences of ePRAI and tPRAI, respectively. Gaps are indicated by dashes. Identical residues between yPRAI and ePRAI or tPRAI are connected by vertical bars. Red boxes indicate the position of cleavage found in the circular permuted variants of TRP1 (Split-TRP[44], Split-TRP[53], Split-TRP[187] and Split-TRP[204]). Residues missing in the mutant TRP[204] are shown in yellow. Circular permuted variants of yPRAI constructed by Luger et al. are marked with turquoise and green boxes, fragments isolated by Eder et al. are labeled in green.

Fig. 4A shows growth results of expression construct in the yeast expression vector pRS316. The green and turquoise bars represent the interacting antiparallel leucine zippers (Z1 and Z2), the gray bars represent the N- and C-terminal TRP1 fragments. Z1-TRP[C] is expressed under the control of the copper promoter

pCUP1, whereas TRP^N-Z2 is expressed under the control of the galactose promoter pGAL1.

Fig. 4B shows the growth results of Split-TRP[44, 53, 187, 204, 77 and 135] and different deletion constructs thereof at 30°C and 23°C. Δ signifies the absence of the corresponding substance in the plates, + means that the corresponding substance has been added to the medium.

Fig. 5A is a schematic representation of two interacting membrane proteins fused to the Split-TRP constructs. Due to the electrostatic interaction of Sec62 and Sec63, the TRP1 fragments (gray "pieces of cake") should come to close proximity and restore active enzyme.

Fig. 5B shows two non-interacting proteins (Sec62 and STE14) are not able to restore TRP1 activity leading to cell death on plates lacking tryptophan.

Fig. 6 shows the growth results of the membrane proteins Sec62, Sec63 and STE14 fused to the different Split-TRP constructs after 7 d incubation at 23°C. Δ signifies the absence, + the presence of the corresponding substance.

Detailed Description of the Invention

A milestone method for the detection of protein-protein interactions in living cells was the yeast-two hybrid system invented by Fields and Song in 1989. Many other approaches, often based on fragment complementation, have been published since then. In general, these systems consist of two fragments or domains of an enzyme or a protein fused to two interacting proteins A and B. Only due to the interaction of A and B the fragments of the protein sensor come to close proximity and the activity is restored. One of the first and until now widely used approaches is the ubiquitin-based split-protein sensor. Other systems use β-galactosidase, DHFR, β-lactamase or EGFP as reporter enzymes. However, despite the constantly expanding repertoire of methods, none is able to answer all question. Often, systems are limited to certain cell types and compartments or exclude some classes of proteins from being investigated. This indicates the need for multiple approaches for the detection of protein-protein interactions in living cells.

One general drawback in the invention of new split sensors is the identification of suitable fragmentation sites in the reporter enzymes or proteins. We therefore used a combination of random circular permutation and homologous recombination to isolate suitable domains. The enzyme of choice is phosphoribosyl anthranilate isomerase (yPRAI or TRP1) from yeast that catalyzes the third step in the biosynthesis of the amino acid tryptophan. As TRP1 deficient yeast strains cannot grow on medium lacking tryptophan, the enzyme is frequently used as selective marker in yeast genetics. TRP1 is a monomer of 25 kD in yeast and has an 8-fold β/α-barrel fold common with about 10 % of all structurally known proteins. The enzyme has been rationally circularly permuted and fragmented before, making of TRP1 a very promising candidate for split-protein sensors.

4

proteins. The enzyme has been rationally circularly permuted and fragmented before, making of TRP1 a very promising candidate for split-protein sensors.

We present here a general approach for the identification of fragmentation sites in a protein and its application to the yeast enzyme TRP1. Randomly generated fragments were initially fused to interacting antiparallel leucine zippers and selected for their ability to complement tryptophan auxotrophy after reconstitution. In addition, a new approach is introduced based on TRP1 as a Split-protein sensor for the detection of protein-protein interactions in living cells. Both, interactions between soluble proteins and membrane proteins can be detected, thus complementing the classical yeast-two hybrid system.

Materials and Methods

Chemicals. Fine chemicals from Alrich, Fluka, Sigma. Amino acid mix from sigma. Single amino acids from Fluka. Enzymes for molecular biology from MBI Fermentas and NEB. Reagents for immunoblotting NEN.

Composition of selective plates.

Selection of Split-TRP sensors. The *trp1* gene coding for the yeast enzyme yPRAI was amplified from pYESTrp2 (Invitrogen) and cloned into a high copy vector using a *Hind*III (RE1) restriction site previously introduced by PCR at amino acid 61 (silent mutation), resulting at the same time in a reorientation of the gene. The plasmid was then amplified and the rearranged *trp1* gene cut out with *Hind*III (RE1). This detour was necessary, as the PCR product, digested with *Hind*III, could not be circularized directly. Only larger oligomers were formed. The isolated *trp1* DNA was circularized by T4 DNA ligase at 16°C for 15 h. Agarose gel analysis revealed that about 5-10 % of the DNA was circularized, the

rest stayed linear or formed dimers and larger oligomers. The whole ligation mixture was randomly digested with DNaseI under conditions to obtain linear monomers. The corresponding band was cut out of the agarose gel. The DNA was blunt ended with a mixture of T4 DNA polymerase/T4 DNA ligase and cloned into a pRS316 derived yeast expression vector and transformed into E. *coli* XL1Blue (*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* (F *proAB lacI^{Q}lacZ ΔM15 Tn10*). The vector already contained two antiparallel leucine zippers that were generated using short oligonucleotides using PCR. They were flanked by an N-terminal FLAG-tag (MDYKDE) and a C-terminal ha-tag and separated by an *Hpa*I restriction site to generate blunt ends after cleavage. After cloning of the library into the blunt ended expression vector, the newly generated N- and C-termini were each connected with one of the antiparallel leucine zippers by a short polypeptide linker (GSGSG and GSGSGG, respectively). The colonies obtained after transformation in *E. coli* were scraped off the plates with 10 % glycerol and stored in aliquots at –80°C. One aliquot was cultivated in LB-medium over night and the DNA extracted by DNA maxi preparation (QIAGEN). Between the original N- and C-termini of *trp1* the unique restriction site *Avr*II (RE2) had been introduced to allow the linearization of the vector and subsequent homologous recombination to separate the gene in fragments. A DNA-fragment containing the CYC1-terminator from the plasmid pYSTrp2 (Invitrogen) and a geneticin resistance followed by the GAL1-promoter from the plasmid pFA6a-GAL1 (Nils Johnsson) was generated by PCR, possessing a 50 base pair homologous region of the N- and the C-terminus of *trp1* at the 3'-and 5'-end, respectively. 300 ng of the linearized plasmid DNA were transformed with 3 μg of the PCR product in chemically competent EGY48 (MATα *ura3 trp1 his3* 6*lex*Aop-*LEU2*) yeast cells in three different reactions and plated on uracil deficient plates containing 500 μg/ml geneticin to select for recombinants. The clones were then analyzed for

6

complementation of tryptophan auxotrophy by plating the colonies on plates lacking tryptophan and uracil (genetic marker) but containing 0.1 mM $CuSO_4$ and 2 % galactose. The C-terminal fragment fused to one leucine zipper (Z1-C) is under the control of the promoter pCUB1 and expressed in the presence of copper, whereas the N-terminal fusion (N-Z2) is expressed from pGAL1. Surviving colonies were selected plated again on different selective plates without tryptophan but containing copper or copper and galactose. Colonies only growing on plates that contain both inducers were characterized by separate PCR amplification of the N- and C- terminal fragment with their corresponding zippers. Constructs that contained more or less wild-type length after the addition of the fragments were analyzed by DNA sequencing.

Membrane Protein Fusions. The genes of Sec62, Sec63 and STE14 were amplified by PCR from yeast EGY48 genomic DNA and combined by overlap extension PCR with the N- and C-terminal fragments of *trp1*, respectively (*4-7*). Split-TRP^N-Sec62 was then subcloned in a pRS315 (Leu$^+$) derived expression vector under the control of pCUB1, whereas Sec63-Split-TRP^C and STE14-Split-TRP^C were cloned into pRS316 (Ura$^+$) under the control of pCUB1. All Sec63 and STE14 fusions contained a ha-tag at the C-terminus of TRP1.

Expression of the Split-TRP^N-Sec62 fusion proteins was confirmed by complementation of the temperature sensitive yeast strain RSY529 (MATα his4 leu2-3,112 ura3-52 sec62-1). Only cells expressing active Sec62 from the additional plasmid were able to grow at the permissive temperature of 37°C. Control cells without a functional copy of Sec62 on the plasmid did not grow. Expression of Sec63 and STE14 fusion proteins was verified by immunoblotting against the ha epitope at the C-terminus of TRP1.

containing 0.1 mM CuSO$_4$ to induce protein expression from the pCUB1 promoter. The plates were incubated at 23°C and 30°C for 4-10 days to observe the growth behavior of the different constructs.

Results and Discussion

Identification of fragmentation sites in TRP1. The first step in the development of a new fragment complementation assay is the identification of a suitable fragmentation site in the protein of interest. As rational approaches often fail, we decided to use a combination of random circular permutation and homologous recombination to reach our aim. Therefore, the *trp1* gene was circularly permuted mainly as described in the literature (Figure 1). After amplification of the *trp1* gene of S. cerevisiae, cleavage with the unique restriction enzyme RE1 and circularization (Fig.1, step 1), the ligation mix was cleaved at random to linearize the DNA (Fig. 1, Step2). The fragments corresponding to the wild-type length were isolated (Fig. 1, Step2) and sub-cloned using blunt ends into the pRS316 based yeast expression vector under the control of a copper promoter (pCUB1) and transformed into E. *coli* XL1Blue (Fig. 1, Step 3). In contrast to the method described in the literature we expressed the protein as two fragments and not as single peptide chain. Therefore, two leucine zippers are located in the plasmid at the 3'- and the 5'- end of the newly generated N- and C-termini. The zippers were designed as positive and negative charged helices to allow heterodimerization due to electrostatic interactions (*4*). Additionally, each of them contained a buried asparagin residue in a position to force antiparallel orientation of the zippers (*4*). The size of the library after transformation in E. *coli* was 3.6 × 10^4 independent clones. Statistically, 50 % of the resulting library has the wrong orientation due to the blunt end cloning, and only one third of them possess the correct reading frame for the expression of both leucine zippers. However, a library size of about 3.6 ×

10^4 should be sufficient to isolate suitable fragmented candidates, even though it is unlikely to generate a complete fragmentation map of TRP1.

In a first attempt to isolate TRP1 fragments, a ribosome binding site was located between the original C- and N-terminus. However, only quasi wild-type enzymes were isolated with random cuts close to the original N- or C-terminus (data not shown). The consequence was a very high background of false-positives, as these quasi wild-type enzymes did not need a second fragment for complementation. To avoid this problem, we combined the circular permutation step with homologous recombination (Fig. 1, step 4). Homologous recombination in yeast has been shown to take place with homologous regions as small as 30-40 base pairs. However, to ensure sufficient recombination 50 base pairs at the N- and the C-terminus, respectively, were chosen. As the fragment to recombine contains the gene for geneticin resistance, only cells that successfully recombined can survive on plates containing geneticin. Consequently, all clones without intact N- and C-termini not able to recombine (i.e. many of the false-positives) should be eliminated.

During the first steps of amplification of the *trp1* gene the unique restriction site *Avr*II (RE2) had been introduced between the original N- and C-termini to allow subsequent linearization of the vector. A fragment consisting of the CYC1-terminator, the geneticin resistance and the GAL1-promoter was generated by PCR, possessing the 50 base pairs homologous region of the N- and the C-terminus of TRP1. Linearized vector (0.3 µg) and PCR fragment (3 µg) were then co-transformed in chemically competent EGY48 and plated on uracil deficient plates containing geneticin to select for recombinants. After 3 d of incubation at 30°C approximately 1600 colonies were growing and subsequently replica-plated on TRP-selection plates lacking uracil and tryptophan but

9

containing geneticin (250 µg/ml), galactose (2%) and copper (0.1 mM) to select for enzyme activity and survival of the cells. 45 colonies were able complement tryptophan auxotrophy. To analyze them further, the cells were streaked out on tryptophan deficient plates containing copper or copper and galactose. Exclusive expression from the galactose promoter was not possible as there are always traces of copper in the medium. Clones growing only in the presence of copper were discarded as false-positives, assuming that one TRP-fragment alone is already able to act as active enzyme. Cells growing only in the presence of both inducers were analyzed further. The approximate size of the fragments was determined by PCR using the plasmid-containing yeast cells as template. 13 clones resulting in more or less wild-type length after addition of the two fragment sizes were sequenced. The results are shown in figure 2.

Five of the 13 clones (Split-TRP[44, 53, 187, 204] and [77]) shown in figure 2 are expressed with two interacting leucine zippers. Seven constructs have only one zipper, indicating no need of interacting proteins for reconstitution of active enzyme. Only one clone is expressed without any zipper at all. This is in agreement with the results of Eder and Kirschner who showed that two TRP1 fragments consisting of amino acids 1-167 and 170-224 could be expressed separately and were able to reassemble autonomously to a functional protein with almost wild-type activity. Four of the 13 clones evolved from a circular DNA molecule (TRP[44, 53, 187] and [135]), whereof three possess two leucine zippers. Most of the clones, however, are derived from dimers or higher oligomers, resulting in overlapping fragments that do not need the leucine zipper interaction for reassembly. Interestingly, in one of the clones fused to both leucine zippers nine amino acids in the middle of the protein are missing (TRP[204]), nevertheless resulting in active enzyme. These

do not need the leucine zipper interaction for reassembly. Interestingly, in one of the clones fused to both leucine zippers nine amino acids in the middle of the protein are missing (TRP[204]), nevertheless resulting in active enzyme. These residues correspond to an additional α-helix of PRAIs (α8') not present in the classical 8-fold β/α-barrels like triosephosphate isomerase (TIM).

Unfortunately, the crystal structure of yPRAI has not yet been solved. Only structures of the homologous enzymes ePRAI and tPRAI that have both about 30 % sequence identity with yPRAI are available. Figure 3A) shows the sequence alignment of the three enzymes and the fragmentation sites of the most promising clones for the use in a fragment complementation assay, Split-TRP[44, 53, 187 and 204] (red boxes). The locations of the cleavage sites in the structure of the homologous enzyme tPRAI are shown in figure 3B).

The mutants Split-TRP[44], Split-TRP[53], Split-TRP[187], Split-TRP[204] were further characterized to assess their value as Split-protein sensors. To this aim, the leucine zipper fused to the N-terminal TRP1 fragment was deleted (Fig. 4, A). As a consequence, the reconstitution of the N-and C-terminal fragment should no longer be possible with only one remaining leucine zipper. Separately, the C-terminal fragment was removed together with its leucine zipper and the remaining N-terminal TRP1 fragment was expressed under the control of the galactose promoter. All constructs were streaked on plates lacking tryptophan and with or without copper and galactose to observe the growth behavior (Figure 4).

As expected, all mutants grow on plates, lacking uracil, indicating simply the presence of the plasmid after transformation into yeast EGY48 cells. Selective plates (no tryptophan) containing copper only allow the growth of construct Split-

TRP[77] (Fig 2, Fig. 4). The protein expressed under the control of the copper promoter lacks the first ten amino acids, which are apparently not necessary for enzyme activity. Under conditions of co-expression of the fragments (in the presence of galactose and copper) growth is only possible for the clones that are in possession of both leucine zippers and both TRP1 fragments. Deletion of one of the zippers (ΔZ2, Fig. 4) prevents cell growth, indicating that reconstitution of the Split-TRP fragments dependent on the interaction of the antiparallel leucine zippers. Deletion of the C-terminus with its corresponding zipper (ΔZ1-C, Fig. 4) and hence exclusive expression of TRP[N]-Z2 equally results in cell death.

Interestingly, two of the clones (Split-TRP[53 and 204]) can only reconstitute TRP1 activity at room temperature (~ 23°C) but not at 30°C. It is as much surprising as the fragmentation site of Split-TRP[44] lies in one of the active site loops between β2 and α2, very close to a conserved arginine in the TRP family. This position would clearly not have been chosen as fragmentation point in a rational approach. The same is true for Glycine 187, which is located in α-helix α7. It is surrounded by many conserved residues, including the two proposed phosphate binding sites of the TRP family. On the opposite site of the enzyme lies the fragmentation site Alanine 53 in α-helix α2, which is extended by nine residues in the yeast enzyme in contrast to TrpF of the bacterial enzymes. The temperature dependence is perhaps more readily understandable in the case of Split-TRP[204], where nine amino acids are deleted following the fragmentation site. This results in a very short C-terminal fragment of only twelve residues, 62 residues together with the leucine zipper. A decrease in temperature could, on one hand, enhance the amount of soluble protein. On the other hand reconstitution is facilitated at lower temperatures, which is indispensable when a peptide fragment is missing in the middle of the protein. Interestingly, the first of the absent amino acids is part of an

additional α-helix α8' in the structures of PRAIs, that is not present in other members of the family of (β/α)₈-barrel enzymes. How can we explain that some fragmentation points do need interacting protein to allow their reconstitution while others are independent of this interaction? One of the 13 sequenced constructs evolved from a circular DNA-molecule and only contains one leucine zipper. Consequently, the two resulting fragments are able to restore active enzyme without the need of interacting proteins. The fragmentation is located on the opposite of the active site in α-helix α5. However, this helix only exists in tPRAI, but not in the enzymes of yeast and *E. coli*. Eder et al. have shown that a large N-terminal fragment of TRP1 consisting of the first 167 amino acids folds independently and possess a defined structure (*6*). In contrast, the shorter C-terminus had to be expressed as a fusion to DHFR to result in soluble protein. After protease cleavage and liberation of the folded C-terminus, both fragments could associate stoichiometrically to form fully active enzyme. Similar to the findings of Eder et al., Split-TRP[135] consists of a larger N-terminal fragment (135 amino acids), which probably folds independently, whereas the smaller C-terminus is expressed as fusion the leucine zipper, assisting in the folding process.

Application of the Split-TRP sensors for the detection of membrane protein interactions. One major disadvantage of the classical yeast-two hybrid system using transcription of a reporter enzyme to detect protein-protein interactions is the limitation of proteins to investigate, as they all have to be transported into the nucleus. Hence, only soluble proteins can be studied that must not be able to activate the transcription of the reporter enzyme by itself. Therefore it would be most desirable to have a new system in hand that can overcome this disadvantage. To this end we tested our new Split-TRP sensors for the ability to detect the interaction of two membrane proteins. Sec62 and Sec63 were chosen as

interacting partners to replace the leucine zippers. Both proteins are located in the membrane of the endoplasmatic reticulum (ER) and are part of the translocation machinery of the ER. Sec62 was fused to the C-terminus of the N-terminal fragment of the Split-TRP[44,53,187and204], whereas Sec63 was fused N-terminal to the corresponding C-terminal amino acids. This arrangement was imposed by the structure of Sec62 and Sec63 and the orientation of the respective termini in the cytoplasm (Fig. 5 A). As a control STE14, like Sec62 and Sec63 a protein of the ER membrane, was fused to the C-terminal fragments of Split-TRP and co-expressed with the corresponding Sec62 fusion proteins (Fig. 5 B).

All fusion proteins, Split-TRP^N-Sec62 and Sec63/STE14-Split-TRP^C, were expressed under the control of a copper promoter on a vector with leucine and uracil as genetic markers, respectively. This should facilitate the same expression pattern and intensity for all fusion constructs. Enzyme activity of TRP1 as a sign of protein interaction was tested on different selective plates lacking tryptophan and the results are shown in Fig. 6.

As can be observed in Fig. 6, Split-TRP^{44} is very well suited to monitor the interaction of the two membrane proteins Sec62 and Sec63. However, activity could only be reconstituted at room temperature, not at 30°C, the common temperature for yeast to grow. Split-TRP187 only yields very small colonies after one week of incubation. Incubation times up to 10 days makes them clearly visible (data not shown). In the case of Split-TRP^{204}, small colonies start to grow only after 10 days. No TRP1 activity at all can be reconstituted when the enzyme is fragmented at position 53. As expected, the negative controls combining Sec62 and STE14, two non-interacting membrane proteins, with the corresponding TRP1 fragments result in cell death. Interestingly, the picture changes when expressing

14

the constructs in yeast cells that do not contain an endogenous copy of Sec62. In this case cells containing plasmid encoded Sec62 and Sec63 fused to the Split-TRP[187] fragments grow faster than when fused to Split-TRP[44]. As one can imagine, space in the membrane is limited and proteins are forced to close contact. This fact could probably facilitate the contact and assist in the reconstitution of the active TRP1 enzyme in the case of Split-TRP[44]. In another scenario endogenous Sec62 could compete with the plasmid encoded one for the interaction sites of Sec63-Split-TRP[C] and hence facilitate the reconstitution in the absence of endogenous Sec62.

Conclusion. Split-protein sensors have become an indispensable tool for the analysis of protein-protein interactions in living cells. The present invention is a combinatorial method for the generation of Split-protein sensors and its application towards the enzyme phosphoribosyl anthranilate isomerase yPRAI/TRP1 from *Saccharomyces cerevisiae*. The generated Split-TRP protein sensors allow for the detection of protein-protein interactions both between cytosolic and membrane proteins by complementing tryptophan auxotrophy in yeast strains. The simplicity of the assay makes Split-TRP an attractive alternative for the currently used Split-protein sensors, in particular for high-throughput applications. The assay is not restricted to yeast but can be also done in all organisms that rely on tryptophan biosynthesis including E. coli or M. smegmatis. We envision also the screening of libraries against a given target as well as the screening of libraries against libraries. The use of TRP1 as a split protein sensor is furthermore not limited to the split sites here introduced or to the enzyme from yeast. In addition, the fragmentation sites identified in the α8β8 barrel structure of TRP1 allow for valuable insides into protein folding.

Claim

A method for the identification of fragmentation sites in a protein using a two hybrid system:

- creating fragmentation sites in TRP1 using gene cleavage with the unique restriction enzyme RE1 and circularization;

- isolating fragments corresponding to the wild-type length;

- sub-cloning using blunt ends into the pRS316 based yeast expression vector under the control of a copper promoter (pCUB1) and transforming into E. *coli* XL1Blue;

- recombining and amplifing homologues with the unique restriction site *Avr*II (RE2) introduced between the original N- and C-termini to allow subsequent linearization of the vector;

- locating two leucine zippers in the plasmid at the 3'- and the 5'- ends of the newly generated N- and C-termini, the zippers being positive and negative charged helices to allow heterodimerization, each heterodimer containing a buried ə .ıragin residue in a position to force antiparallel orientation of the zippers.
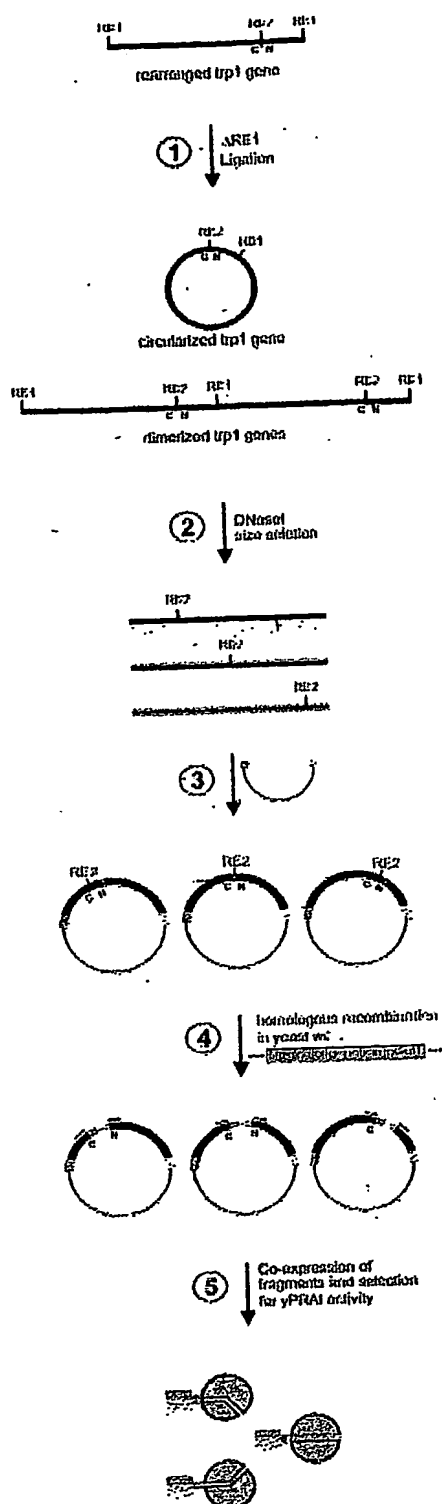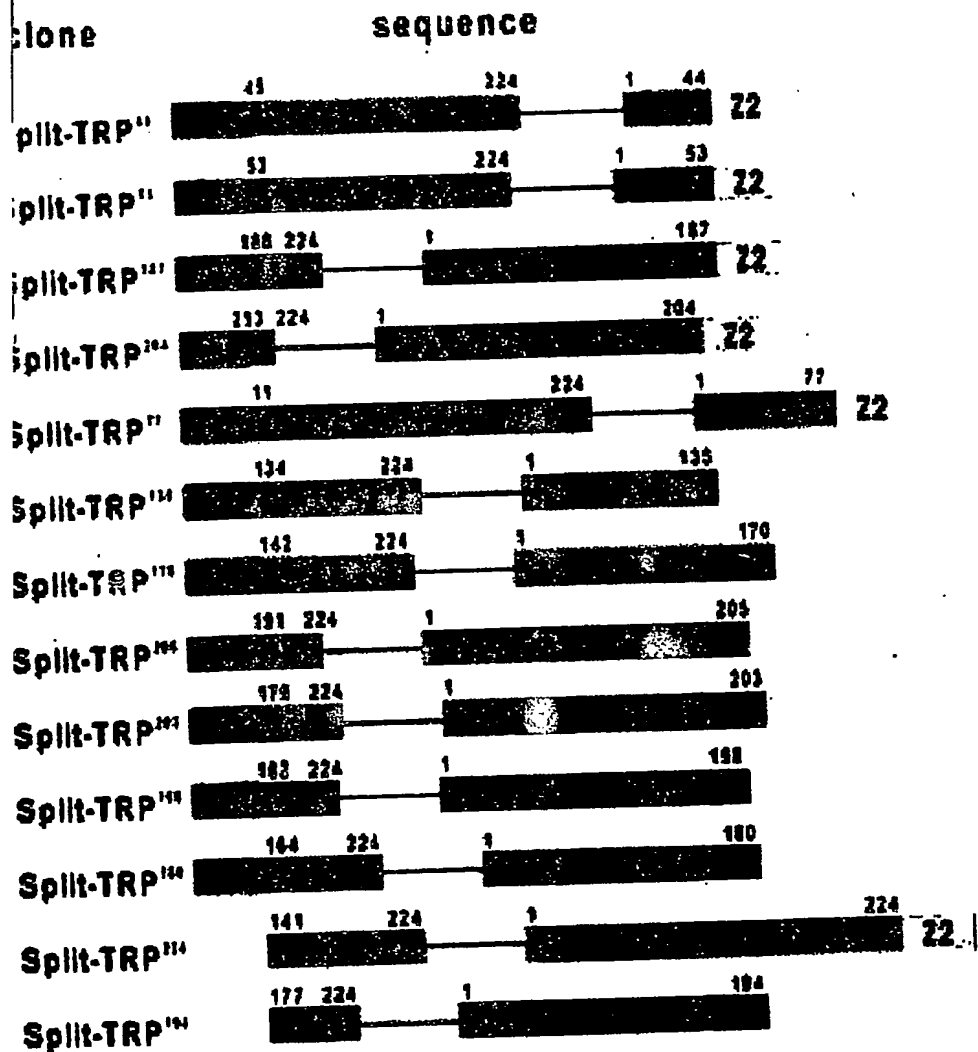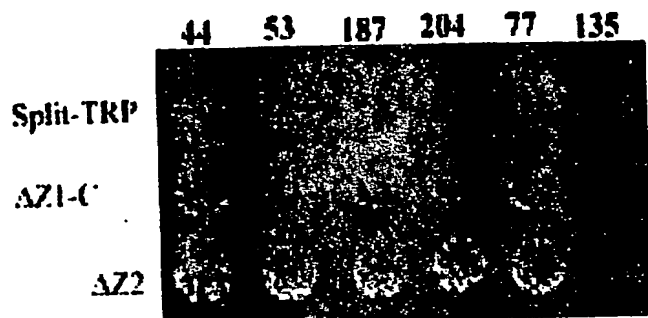
Figure 1

FIGURE 2

Figure 3

```
ePRAI  -----RVLL--GENKVCGLTRGQDAKAAYDAGAIYGGLIFVAT PRCVNV  293
yPRAI  MSVINFTGSSGPLYKVCCLQSTEAAECALDSDADLGIICVPN KRTIDP   50
cPRAI  -------MVRVKICGITNLEDALFSVESGADAVGFVFYPK KRYISP     40

ePRAI  EQ QEV-------PLQYVGVFRNRDIADVVDKARVLSLAAVQ         332
yPRAI  VI RKISSLVKAYKNSSGT--PKYLVGVFRNQPREDVLALVNDYGIDIVQ  98
cPRAI  ED RRI-------SVELPPFVPRVGVFVNEEPEKILDVASYVQLNAVQ    81

ePRAI  LHGNEEQLYIDTLREALPARVAINKALSVG---ETLPAREFQHVDKYVL  379
yPRAI  LHGDESMQEYQBFLGLPVIKRLYPPKDCNI---LLSAASQKPHSFIPLF  145
cPRAI  LHGEEPIELCRKIAER----ILVIKAVGVSNERDMERALNYREF-PILL  126

ePRAI  NGQ---GGSGQRFDMSLL------NGQSL--GNVLLAGGLGADNC ERAA  417
yPRAI  SEA----GGTGELLDWNSISDWVGR ESPES--LHFMLAGGLTPENVDAL  190
cPRAI  TKTPEYGGSGKTFDMSLI------LPYRDRFRYLVLSGGINPENV SAI  169

ePRAI  Q-TG-CA LDENSA ... QPGKDARLLASVFQTLRAY  452
yPRAI  RLNG-VIGVDYS G ... NKIANFVKNAKK-        224
cPRAI  D-VVRPFAVDVSSG ... DSIKMFIKNAKGL        206
```

Split-TRP pCUP1 ▬▬▬ pGAL1 ▬▬▬ Z2

30°C

Δ uracil
– tryptophan
– copper
Δ galactose
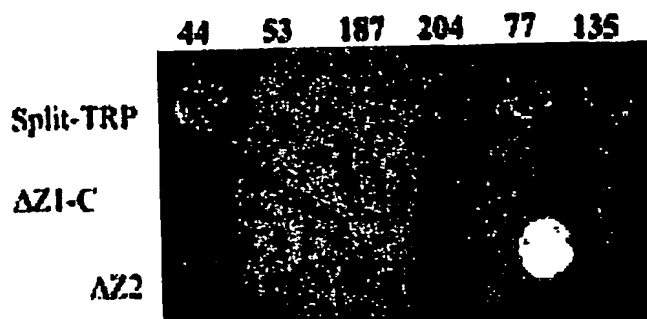
Δ uracil
Δ tryptophan
+ copper
Δ galactose

Δ uracil
Δ tryptophan
+ copper
+ galactose
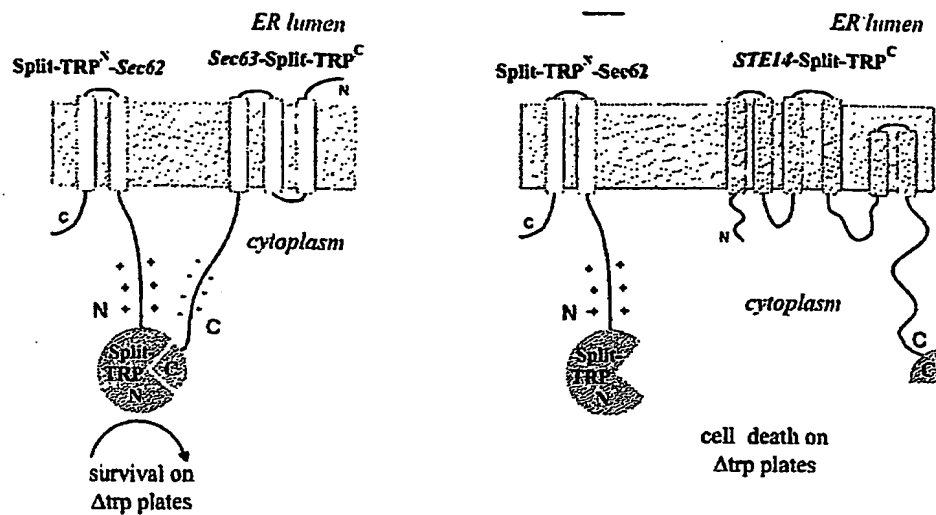
23°C

Δ uracil
Δ tryptophan
+ copper
– galactose

FIGURE 4

ER lumen

Split-TRP^N-Sec62   Sec63-Split-TRP^C

cytoplasm

N

C

Split-TRP N   C

survival on
Δtrp plates

ER lumen

Split-TRP^N-Sec62   STE14-Split-TRP^C

N

cytoplasm

N

C

Split-TRP N

C

cell death on
Δtrp plates

Figure 5

Split-TRP^{44} -Sec62/63	Split-TRP^{53} -Sec62/63	Split-TRP^{187} -Sec62/63	Split-TRP^{204} -Sec62/63
Split-TRP^{44} -Sec62/STE14	nd	Split-TRP^{187} -Sec62/STE14	nd
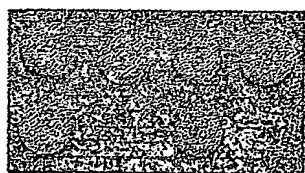
Δ leucine
Δ uracil
+ copper
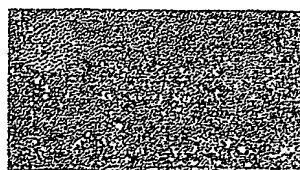
Δ leucine
Δ uracil
Δ tryptophan
+ copper

Figure 6